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GABA UPTAKE BY GAT1 MODULATES LONG-TERM OPTICAL CHANGES FOLLOWING ELECTRICAL STIMULATION OF THE PITUITARY GLAND NEUROINTERMEDIATE LOBE

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Intrinsic optical changes that follow infundibular stalk stimulation of the neurointermediate lobe of the mouse pituitary gland exhibit three different phases that reflect three distinct physiological events. The first (E-wave) is the rapid light-scattering increase that is associated with a nerve terminal volume increase (mechanical spike), and that accompanies excitation of the neurohypophysial terminals by the invading action potential; the second (S-wave) is the slower light-scattering decrease that is tightly correlated with the secretion of the peptide hormones oxytocin and arginine vasopressin, and the third is the long-duration response (R-wave) that reflects cell volume changes in the *pars intermedia*. We have studied the E-wave and the S-wave in earlier publications. The R-wave, considered here, is sensitive to chloride replacement as well as to blockade of chloride channels. By blocking $GABA_A$ receptors (which are ligand-gated chloride channels) with pharmacological agents, and by applying GABA directly into the bathing solution, or evoking its release from GABAergic inputs, we have demonstrated that this long-duration optical response is sensitive to chloride movements and reflects GABA-induced changes in the intrinsic optical properties of the *pars intermedia*. The full time-course of this optical response takes minutes and, therefore, has to embody some other process (or processes) related to the restoration of resting physiological chloride concentrations, following the opening and closing of $GABA_A$-receptor channels. Here we demonstrate that the shape of the R-wave, the long-lasting

light-scattering signal, is indeed affected by the activity of GAT1, one of the sodium- and chloride-dependent GABA transporters.

Keywords: Light scattering; posterior pituitary; *pars nervosa*; neurohypophysis; volume changes; *pars intermedia*; $GABA_A$ receptors.

1. Introduction

The hypothalamus is an unusual part of the brain in that it is one of the sites where electrical signals in neurons are converted to humoral ones. From the hypothalamus, magnocellular neurons project their axons through the median eminence and the infundibular stalk to terminate in the neurohypophysis (also known as posterior pituitary or *pars nervosa*), a neuroendocrine gland whose secretory terminals and swellings release the peptide hormones arginine vasopressin and oxytocin. In addition, GABAergic hypothalamic neurons, which also project their axons through the infundibular stalk, terminate at the border between the neurohypophysis and the *pars intermedia*, where they make contact with *pars intermedia* cells.[1,2] In this way, GABA, secreted in response to infundibular stalk stimulation, is capable of modulating activity in the *pars intermedia*.[3,4]

The intrinsic optical changes associated with neurointermediate lobe stimulation, first observed in our laboratory in 1985,[5] now include three components that are associated with three distinct physiological events. The first one is a rapid light-scattering increase, manifested as a decrease in transmitted light intensity (E-wave) resulting from a rise in turbidity, due to an increase in nerve terminal volume (mechanical spike) coincident with excitation of the terminals by an invading action potential[5,6]; the second intrinsic optical change is the slower decrease in light scattering (S-wave) that reflects secretion of the peptide hormones oxytocin and arginine vasopressin,[5,7–9] and the third one is the long-duration response (R-wave).[10] It is this R-wave that is the focus of the present work. Using mechanical, optical and pharmacological interventions, we were able to confirm that, while the first two phases of the light-scattering change, the E- and S-waves, originate in the neurohypophysis, begin immediately after the electrical stimulation, and can be measured in milliseconds,[5] the R-wave reflects GABA-induced changes in the intrinsic optical properties of the *pars intermedia*, rather than those of the neurohypophysis itself,[10] and also is sensitive to chloride replacement by less permeable anions.[10,11] Here, we consider the effects of the GABA transporter, GAT1, on the shape of the R-wave.

The term "R-wave" had its origin in our initial observation several years ago (limited to ≤ 4 s data acquisitions), that the component of the light-scattering signal that follows the cumulative S-wave triggered by a train of action potentials, always included a downward sloping portion, which suggested a return to baseline. That slope, although invariably pointing downward at the end, exhibited variations both in its time-course and in its magnitude. Furthermore, longer recordings revealed that its shape is biphasic, and that, *in toto*, it lasts for several minutes. Now we understand most of its features, as we have determined that (a) the so-called R-wave has its origin exclusively in the *pars intermedia*[10]; (b) the differing relative contributions of neurohypophysial terminals and *pars intermedia* cells to the light-scattering changes in this tissue, in which the physical separation of the two components of the neurointermediate lobe is almost impossible, explains why the shape of the R-wave, until now, varied so widely from experiment to experiment[10]; and (c) the downward phase reflects a volume change in the cells of *pars intermedia*, associated with the opening of $GABA_A$-receptor channels following electrical stimulation of the infundibular stock and the concomitant release of GABA from axonal projections of GABAergic hypothalamic neurons. The aim of the present work is to identify the genesis of the second, ascending phase of the R-wave. But, before we describe the experiments designed to address this issue, we need to acknowledge that the name "recovery-wave" (R-wave) is inappropriate. Since we previously referred to this component as the R-wave,[10] we propose simply to continue this designation without qualification and, more importantly, without the "recovery" implication.

The full time-course of the R-wave takes minutes, and therefore cannot be merely due to chloride-channel opening/closing; instead, as has been

suggested before,[10] it may involve one or more additional processes capable of slowly changing the refractive index and/or the transparency/turbidity of the tissue. Here, we demonstrate that the time-course of this long-duration light-scattering signal, the R-wave, is indeed affected by the activity of GAT1, one of the sodium- and chloride-dependent GABA-transporters, and that this molecule, rather than being ubiquitously distributed in all of the *pars intermedia*, is confined to only a subset of its cells.

2. Methods

2.1. *Tissue preparation*

Details of the neurointermediate lobe dissection have been reported previously (e.g., Refs. 5, 7, 10, 12−15). Briefly, the neurointermediate lobe (comprising neurohypophysis and *pars intermedia*) was obtained from either CD-1 or C57/black mice in the following manner: a female mouse, 30−40 days old, was anesthetized by CO_2 inhalation and decapitated according to institutional guidelines. The head was pinned to the bottom of a Sylgard-lined dissection dish and the skin was removed from the skull. The skull was then opened along the dorsal midline and removed bilaterally. The brain was reflected caudally and pulled out, after cutting optic and olfactory nerves under a low-power dissecting microscope. During this procedure, the infundibular stalk was automatically ruptured, leaving an infundibular stump and the entire pituitary gland (*pars distalis*, *pars intermedia and pars nervosa*) in the base of the skull, held in place by a thin layer of connective tissue. Oxygenated normal mouse Ringer's solution (in mM: 154 NaCl, 5.6 KCl, 1 $MgCl_2$, 2.2 $CaCl_2$, 10 glucose, 20 HEPES, adjusted to pH 7.4 with NaOH) was circulated over the preparation during the removal of the gland, which was performed using iridectomy scissors and fine forceps. Once the whole gland was isolated, the anterior pituitary (*pars anterior*, also known as the *pars distalis*) was readily separated from the neurointermediate lobe (neurohypophysis or *pars nervosa*, and *pars intermedia*). The *pars intermedia* itself, consists of a delicated arrangement of cells cradling the neurohypophysis from its ventral side. In the mouse, the complete surgical separation of the *pars intermedia* from the neurohypophysis is virtually impossible to achieve.

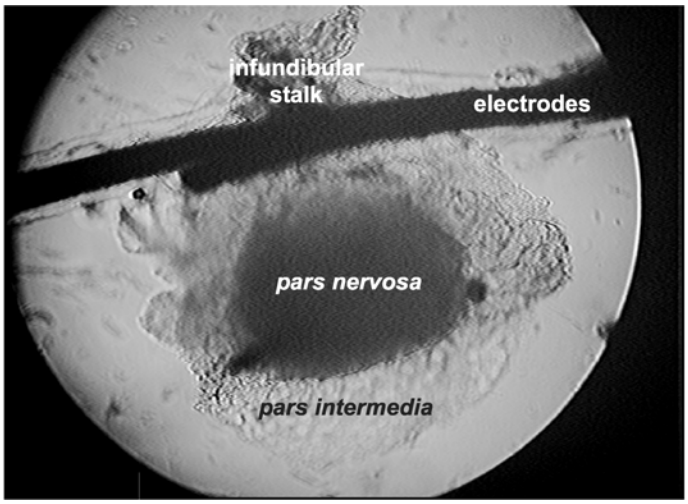
2.2. *Experimental protocol for physiological experiments: Electrical stimulation and optical recording*

A description of the basic apparatus used for the physiological experiments has been reported already (e.g., Refs. 5, 7, 10, 12−15). In short, the isolated neurointermediate lobe was pinned down on the thin Sylgard bottom of a simple chamber filled with normal mouse Ringer's, and the infundibular stalk was clasped between a pair of Pt−Ir electrodes coated with Teflon but bared in the narrow region where they contacted the infundibulum [as shown, e.g., in Figs. 1(a)−1(c)]. After resting the preparation for approximately 15 min, brief, balanced bipolar shocks (40−100 V, 300−500 μs in duration), were delivered through a stimulus isolator. The resulting changes in transmitted light intensity were recorded by a single, large-area silicon PIN-photodiode (PV-444, Perkin Elmer Optoelectronics, Vaudreuill, Canada) positioned in the image plane of a large, mechanically stable compound microscope (UEM, Zeiss, Inc., Thornwood, N.Y.). Since the transmitted light ($\lambda = 670$nm) was collected with an objective of high numerical aperture (HCX APO; 20X; 0.5 N.A.; Leica, Inc., Germany), changes in transmitted light intensity represented changes in large-angle light scattering. In addition, an iris diaphragm, located in front of the photodiode, allowed us to choose the optimal size and position of the optical field from which light-scattering changes were obtained. The photocurrent was converted to voltage using a custom made current-to-voltage converter (Yale Dept. of Cellular and Molecular Physiology Electronics Shop, New Haven, CT). All the experiments were carried out at room temperature. All chemicals and pharmacological agents employed (Analytical Grade) were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Physiological experiments were conducted at room temperature ($\sim$23°C). Data acquisition and analysis were accomplished using IGOR software (WaveMetrics, Portland, OR). Traces were corrected for baseline drift, by subtracting the linear extrapolation of the initial baseline segments.

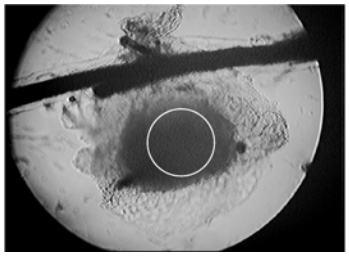
2.3. *Tissue preparation for immunocytochemical assays*

Immunofluorescence experiments were performed on mouse neurointermediate lobes fixed as whole

mounts. Freshly dissected neurointermediate lobes were washed in normal mouse Ringer's solution and immediately fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA, USA) for 24 h at 4°C. Following fixation, all the tissue samples were washed (3 times, 60 min each) with Phosphate Buffered Saline/NaN_3 solution (in mM: 10 NaN_3, 10 Na-Phosphate Buffer, 100 NaCl; pH 7.5) at room temperature. Since the antisera used to detect the presence and localization of GAT1 were raised in guinea pig against the N-terminus of the protein, and since that segment is presumably intracellular in the mouse, as it is in the rat (e.g., see Ref. 16), the samples were permeabilized by incubating them for 2 h at room temperature in Phosphate Buffered Saline/NaN_3 containing 0.1% triton and 50 μM EGTA. Following permeabilization, the samples were washed 3 times (20 min each) with Phosphate Buffered Saline/NaN_3, and then incubated overnight at 4°C, with Phosphate Buffered Saline/



(a)



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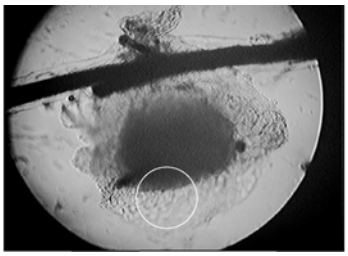

(c)

Fig. 1. Light-scattering signals measured as changes in transmitted light intensity, and recorded at $\lambda = 670$ nm, in the neurointermediate lobe of a mouse pituitary gland upon electrical stimulation of the infundibular stalk. (a) Micrograph of a mouse neurointermediate lobe mounted in the experimental chamber, which shows the different regions of the preparation and the stimulating electrodes clasping the infundibular stalk. 10X magnification. (b) The white ring identifies a region of interest (ROI) almost exclusively restricted to the *pars nervosa*. In response to electrical stimulation of the infundibular stalk, the light-scattering signals from this region will include the E- and S-waves that characterize the neurohypophysial terminals [as in panels 1(d) and 1(f)]. (c) The white ring identifies here a ROI that includes portions of both *pars nervosa* and *pars intermedia*; light-scattering signals collected from this field of view will include E-, S- and R-waves [as in panels 1(e) and 1(g)]. (d) Rapid decrease in transmitted light intensity (E-wave) in response to a single stimulus, which indicates the arrival of the action potential and/or action currents at the secretory terminals of the neurohypophysis (also known as *pars nervosa* or posterior pituitary), followed by the S-wave, an increase in transmitted light intensity tightly correlated to hormone secretion by the same activated secretory terminals; single stimulus, 100 V, 500 μs. (e) Another record, also in response to a single stimulus, displayed on a contracted time base to show the long-duration response (R-wave). (f) Consecutive E-waves and S-waves in response to a train of 7 electrical stimuli (500 μs duration, at 15 Hz for 410 ms). (g) Cumulative R-wave following a train of stimuli identical to that in (c). In order to observe simultaneously the E-wave, the S-wave and the R-wave, the fields of view from which the records in 1(e) and 1(g) were acquired included *pars nervosa* as well as *pars intermedia*, as shown in 1(c).

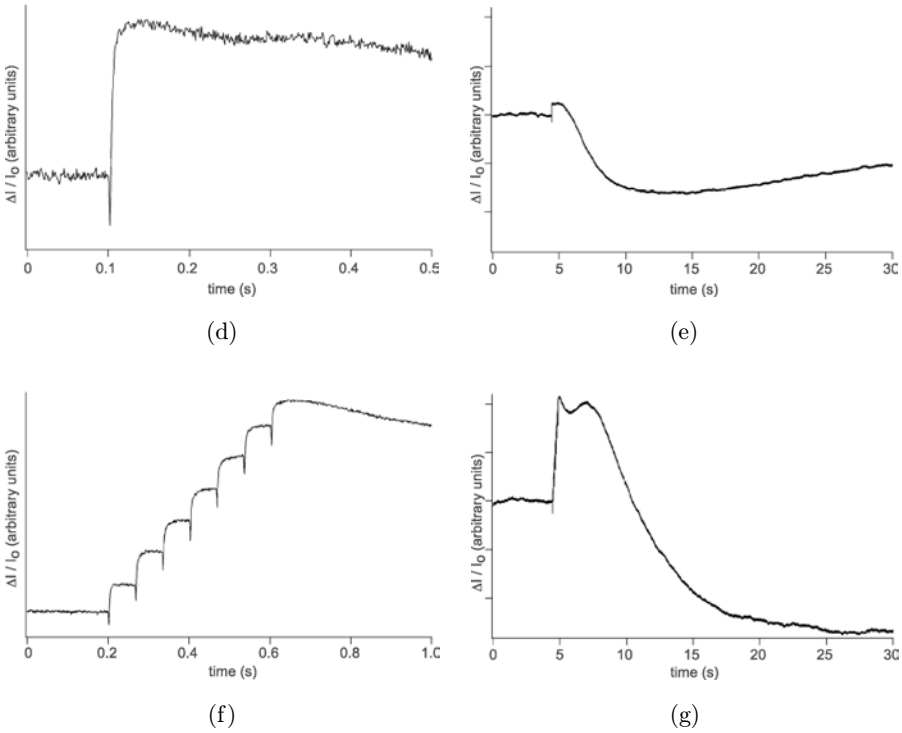

Fig. 1. (*Continued*)

NaN_3 containing 4% normal goat serum (Jackson ImmunoResearch, West Grove, PA, USA) to block nonspecific binding. After that, the samples were ready to be exposed to the immunocytochemical reagents. The protocol included:

Sample A: Blank, neither primary antiserum nor secondary antibody.
Sample B: Control, only secondary antibody.

Samples C and D: Different batches of antiserum to N-terminus of mGAT1 (each batch from a different guinea pig, from within a pair of animals injected simultaneously with the same antigen as indicated in Ref. 17), and secondary antibody.

Primary antisera were applied for 20 h at 4°C, at a 1:50 dilution in Phosphate Buffered Saline/NaN_3 containing 4% normal goat serum. After incubation, the samples were washed 3 times (60 min each) with Phosphate Buffered Saline/NaN_3, and subsequently incubated with the secondary antibody, an Alexa Fluor 488 Goat Anti-Guinea Pig IgG (H+L), highly cross-adsorbed, from Molecular Probes (A-11073, Life Technologies, Grand Island, NY 14072), diluted in the same Phosphate Buffered Saline/NaN_3 containing 4% NGS, up to a final concentration of 2 μg/mL.

After washing the secondary antibody (3 times, 30 min each), the samples were left to dry, and then were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA 94010).

3. Results

Changes in volume of cells and/or of organelles (e.g., Ref. 18), phase changes in localized cytoplasmic regions in response to Ca^{2+}-release from intracellular stores,[19–21] or vesicle dynamics (exocytotic and/or endocytotic events), may affect the refractive index and transparency of a tissue, which can be measured, in turn, as light-scattering changes.[22] Release of their content by dense core secretory vesicles in the *pars nervosa* of the pituitary gland, for example, not surprisingly, shows a tight correlation with mechanical events (e.g., "dip" of the mechanical spike[6] as well as light-scattering changes such as the S-wave[5]).

Figure 1(a) illustrates the anatomical configuration of the mouse neurointermediate lobe as mounted in the experimental chamber, and the position of the electrodes used for tissue stimulation. Figure 1(b) shows, circumscribed by the white ring, a typical field of view dominated almost exclusively by *pars nervosa*. Although the *pars intermedia* is still present underneath the *pars*

nervosa, its thickness in that region is very much reduced, and its position is quite far from the typically chosen focal plane. Therefore, its contribution to the light-scattering changes triggered by electrical stimulation of the infundibular stalk is negligible compared to the light-scattering changes that originate from the millions of neurohypophysial terminals activated synchronously [e.g., Figs. 1(d) and 1(f)]. Figure 1(c), on the other hand, highlights a field of view that includes both *pars nervosa* and *pars intermedia*. Accordingly, upon stimulation, this portion of the preparation will generate light-scattering signals that will include E-, S- and R-waves [as shown in Figs. 1(e) and 1(g)]. For convenience, these light-scattering signals are measured as changes in transmitted light intensity at 670 nm (opposite sign to changes in large-angle light scattering). These regional transitions were explained in detail by Kosterin *et al.*,[10] where we demonstrated unequivocally that while the E- and S- waves have their origin in the neurohypophysis, the R-wave has its origin in *pars intermedia*. Panel 1(d), for example, shows a single E-wave (the rapid decrease in transmitted light intensity or rapid increase in large-angle light scattering) which represents the arrival of the action potential and/or action currents at the neurohypophysial terminals, followed by the S-wave, an increase in transmitted light intensity (or decrease in large-angle light scattering) tightly correlated with secretion from the activated terminals. Notice that both of these signals can be measured in milliseconds. Panel 1(e) emphasizes, instead, the time-course of the R-wave that follows the E-and S-waves generated in response to a single stimulus, and that, unlike the fast neurohypophysial signals, can only be measured in seconds. It is this R-wave that is the focus of the present work.

When the infundibular stalk is shocked using a train of multiple stimuli instead of a single stimulus, a slight variation in the sizes of the individual S-waves and E-waves, and a significant enhancement of the cumulative R-wave, take place. This is illustrated in Figs. 1(f) and 1(g). Since all of these signals, although independent of one another as to their origin, are recorded simultaneously along a single time axis, it is extremely difficult to interpret their variation mechanistically as Obaid and Salzberg[23] had done for the combination of E-wave and S-wave alone in the presence of 4-aminopyridine. We present these data, however, because all the physiological experiments that follow (see Figs. 2–5) used the same protocol as Figs. 1(f) and 1(g), including seven stimuli.

If GABA acting on $GABA_A$ receptors in *pars intermedia* is responsible for the downward component of the R-wave as demonstrated previously (e.g., see Fig. 9 of Ref. 10), the rebound of the signal [e.g., Fig. 1(e)] suggests the possibility that GABA transporters could also be playing a role. To separate the contributions of the GABA-gated Cl^--channel activation from those of putative GABA transporters, we recorded light-scattering changes generated from (mostly, but not exclusively) *pars intermedia* [as illustrated in Fig. 1(c)] while blocking, alternatively, $GABA_A$ receptors or GABA-transporters.

Figure 2 shows the light-scattering changes induced by infundibular stalk stimulation in the presence of GABA itself and of NO711 hydrochloride (NO711),[24,25] an inhibitor of GAT1. It is apparent that block of the transporter with NO711

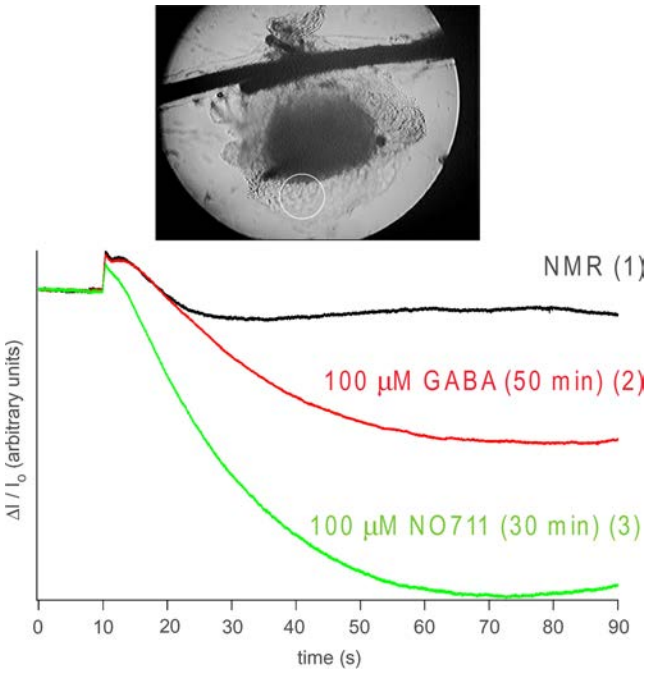

Fig. 2. GABA and NO711 (an inhibitor of GAT1)[25] enhance the downward component of the R-wave evoked by electrical stimulation of the neurointermediate lobe of the mouse pituitary gland, and delay its rebound phase. The upper panel highlights the field of view from where the light-scattering signals were collected. As in Fig. 1, in order to observe simultaneously the E-wave, the S-wave and the R-wave, the field of view from which these records were acquired included *pars nervosa* as well as *pars intermedia*. The numbers in parentheses indicate the order in which the records were acquired. Stimulation consisted of a train of 7 stimuli (15 Hz, 500 μs).

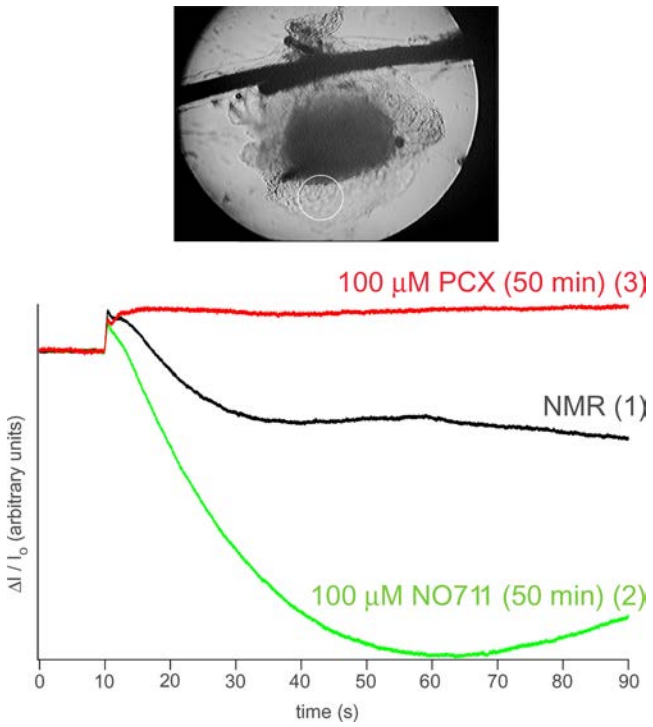

Fig. 3. Effects of blocking GAT1 first with NO711, and then applying picrotoxin (PCX), on the light-scattering signals evoked in the neurointermediate lobe by electrical stimulation of the infundibular stalk. The upper panel highlights the field of view from which the light-scattering signals were collected. As in previous figures, in order to observe simultaneously the E-wave, the S-wave and the R-wave, the field of view from which these records were acquired included *pars nervosa* as well as *pars intermedia*. The numbers in parentheses indicate the order in which the records were acquired. Stimulation consisted of a train of 7 stimuli (15 Hz, 500 μs). NMR is Normal Mouse Ringer's solution. PCX is picrotoxin.

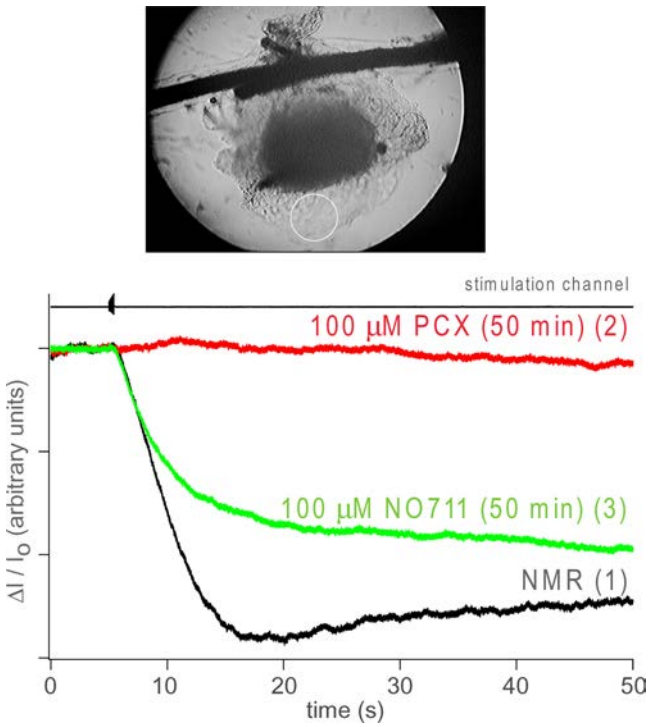

Fig. 4. Light-scattering changes under conditions of consecutive pharmacological blockade of first the $GABA_A$ receptors with picrotoxin (PCX), and then the GAT1 transporter with NO711. The upper panel highlights the field of view from which the light-scattering signals were collected. This record was acquired from a region restricted to *pars intermedia*, and therefore the E-wave and the S-wave are not present. The increased noise of the traces is caused by vibration of *pars intermedia* that has not been pinned in order to fully preserve its integrity. Furthermore, the greater transparency of the *pars intermedia* compared to that of *pars nervosa* lets much more light through the tissue and its vibration amplifies the noise. In this experiment only, we used a light emitting diode (LED; $\lambda = 650$ nm) instead of the 100 W tungsten-halogen lamp used in all the other experiments. Numbers in parentheses indicate the order in which the records were acquired. Stimulation consisted of a train of 7 stimuli (15 Hz, 500 μs). NMR is Normal Mouse Ringer's solution. PCX is picrotoxin.

enhances the downward component of the R-wave in a manner similar to that of the direct application of GABA (Fig. 9 of Ref. 10, suggesting strongly that, if GABA-evoked Cl^- movement causes the swelling of *pars intermedia* cells, GAT1, by reducing available extracellular GABA, tends to reverse that effect.

Figure 3 shows the effect of blocking, first GAT1 with NO711 and, subsequently, all the $GABA_A$ receptors with the $GABA_A$-channel blocker picrotoxin. The inhibition of the transporter, and that of the $GABA_A$ receptors, produce essentially opposite effects on the R-wave: the inhibition of the transporter (green trace) enhances the initial slope of the R-wave by slowing its rebound, while blocking the GABA-gated Cl^--channels (red trace) diminishes or eliminates the downward component of the R-wave.

Figure 4 illustrates the light-scattering changes after sequential pharmacological block of, first the $GABA_A$ receptors (GABA-gated Cl^--channels), and then the transporter. NO711, the GAT1 transporter blocker, produces an effect (green trace), even when most of the $GABA_A$ receptors have been blocked by picrotoxin. The fact that NO711 (at 100 μM concentration) can reverse, albeit partially, the effect of 100 μM picrotoxin, seems to suggest that this concentration of picrotoxin is not supramaximal in this tissue. These records were obtained from a field of view focused exclusively on *pars intermedia* cells,

explaining the absence of the signals that originate in *pars nervosa* (E-wave and S-wave).

The comparison of the results from Figs. 3 and 4 demonstrate that consecutive pharmacological block of the $GABA_A$ receptors and the GAT1 transporter produces opposite effects on the R-wave of the light-scattering signal. Indeed, the activation of the channel (with GABA) induces a light-scattering change (R-wave) similar to that produced by blocking of the transporter.

We thought it would be interesting to examine whether or not we could affect both the Cl^--channel ($GABA_A$ receptor) and the transporter (GAT1) with the same drug. To test this, we chose nipecotic acid,[26] which at low concentrations inhibits GABA uptake, but at high concentrations behaves as a $GABA_A$ receptor agonist. Figure 5 illustrates the effects of applying two different concentrations of nipecotic acid, 100 μM and 4 mM. At 100 μM, this agent clearly inhibits GABA uptake; at 4 mM, its effect resembles that obtained in the presence of the externally applied GABA (shown in Fig. 2, red trace), is opposite to that of picrotoxin (shown in Fig. 3), and is much larger than would be expected from block of the transporter alone.

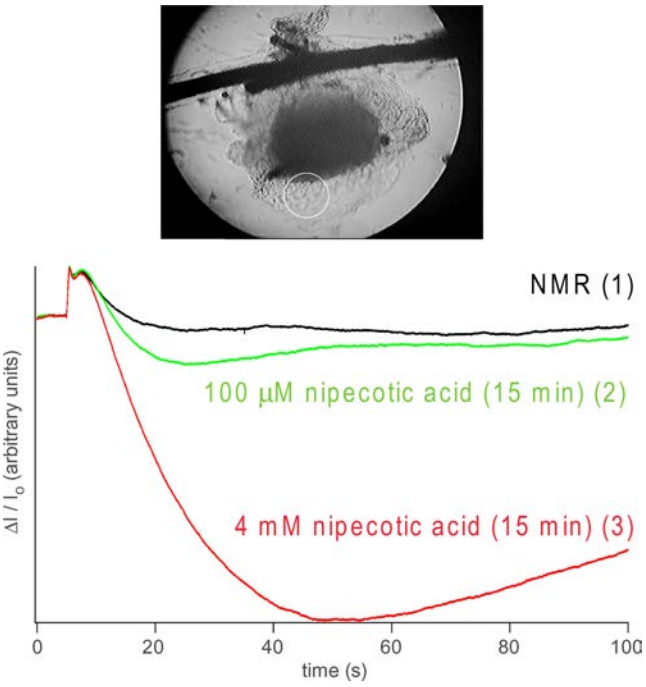

Fig. 5. Light-scattering changes following consecutive applications of nipecotic acid[26] at 100 μM and 4 mM, respectively. The upper panel highlights the ROI from which the light-scattering signals were collected. As in previous figures, in order to observe simultaneously the E-wave, the S-wave and the R-wave, the field of view from which these records were acquired included *pars nervosa* as well as *pars intermedia*. The numbers in parentheses indicate the order in which the records were acquired. Stimulation consisted of a train of 7 stimuli (15 Hz, 500 μs). NMR is Normal Mouse Ringer's solution.

The physiological experiments presented so far demonstrate that GAT1 is present in the mouse neurointermediate lobe. What has not been determined up to now is where in the neurointermediate lobe GAT1 is expressed. The close contact between *pars nervosa* and *pars intermedia*, and the fact that GABAergic inputs from the hypothalamus are found preferentially at the interface between the two tissues (see, e.g., Refs. 1, 2, 27 and 28) and seem to penetrate both (*pars nervosa* shows GABAergic endings around pituicytes and blood vessels, while in *pars intermedia* they appear to make contact with secretory cells), raise the possibility that part of the GABA released onto *pars intermedia* cells could be captured by GAT1 found in neighboring regions of the neurohypophysis. To answer that question we performed immunocytochemical experiments using antisera which had been raised in guinea pig against mouse GAT1, in Dr. Nathan Nelson's laboratory (mGAT1, Ref. 17). The results, shown in Fig. 6, prove that GAT1 is overwhelmingly expressed in *pars intermedia*, revealing clearly that *pars intermedia* is the site of action of NO711 and nipecotic acid in the mouse neurointermediate lobe.

4. Discussion

In the secretory process in the mouse neurohypophysis, cell surface and volume changes caused by the release of the contents of dense-core granules (secretory vesicles), and by the associated flow of water, are rapid events [(Refs. 29 and 30), and also Figs. 1(d) and 1(f)]. With a specially designed apparatus (a High Bandwidth Atomic Force Microscope Ref. 6) capable of registering nanometer scale movements with fast time resolution, these rapid changes can be observed directly.[6] In *pars intermedia*, however, where the cells are much bigger than neurohypophysial terminals and the secretion takes place more slowly, the cell volume changes still affect the refractive index and/or transparency of the tissue, and can also be observed optically as light-scattering changes (R-wave) [see Ref. 10, and Figs. 1(e) and 1(g)]. In addition, we have shown (see Ref. 10, and Fig. 2) that GABA modulates the shape of the downward phase of the R-wave, the much longer-lasting intrinsic optical signal, and

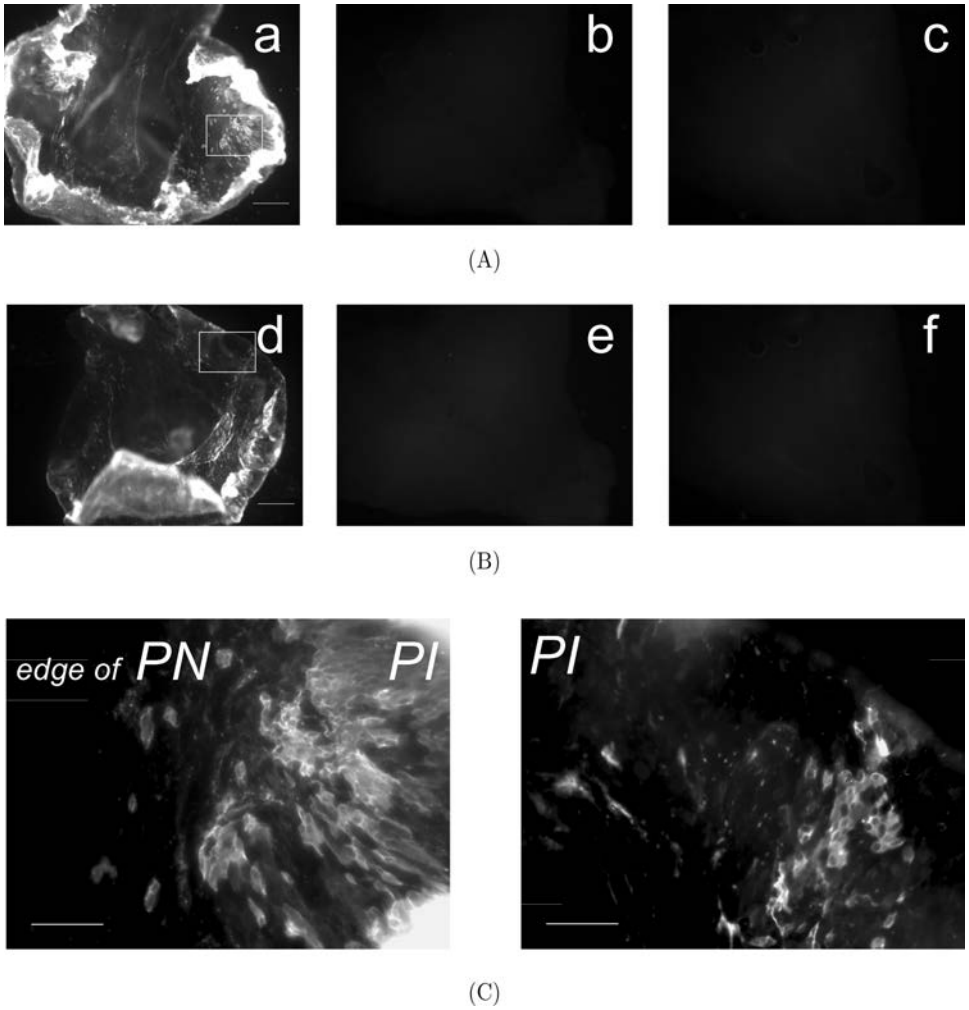

Fig. 6. The pattern of immunoreactivity obtained with mGAT1[17] in the mouse neurointermediate lobe demonstrates that GAT1 is predominantly expressed in a discrete population of cells of the *pars intermedia*. (A) and (B) The expression of GAT1 is mostly confined to *pars intermedia*. (Aa) and (Bd) Mouse neurointermediate lobes, each stained with nonpurified antisera from one of two guinea pigs immunized against the N-terminus of mGAT1, and Alexa 488-conjugated goat-anti-guinea pig IgG. Fixed tissue was permeabilized with Triton X-100 (0.1%). 10X magnification. Calibration bar: 200 μm. (Ab) and (Be) Negative controls for (Aa) and (Bd), respectively, obtained from mouse neurointermediate lobes in the absence of mGAT1. 10X magnification. (Ac) and (Bf) Blanks, obtained from mouse neurointermediate lobes in the absence of both mGAT1 and Alexa 488-conjugated goat-anti-guinea pig IgG. 10X magnification. (C) mGAT1-immunoreactivity is confined to a subpopulation of *pars intermedia* cells. Left and right panels show, respectively, the magnified views of the selected areas as shown in (Aa) and (Bd), revealing that GAT1 immunoreactivity appears in some cells of the *pars intermedia* but is absent in *pars nervosa*. 40X magnification. Calibration bar: 50 μm.

that the $GABA_A$ blockers picrotoxin and bicuculline eliminate this GABA effect (see Ref. 10, and Figs. 3 and 4). Parallel experiments, in which we substituted different impermeable anions (e.g., isethionate or gluconate, see Ref. 10) for the chloride in the bath, however, enhance instead of abolishing the downward phase of the R-wave, indicating that not all of the Cl^- channels in this tissue are activated by GABA, and that the light-scattering signal observed in the presence of GABA is specific to this neurotransmitter. These experiments, together, establish that: (a) GABA-induced changes in the intrinsic optical properties are confined to the *pars intermedia*, while the E- and S-waves originate in the *posterior pituitary* and do not exhibit the same GABA-sensitivity; (b) the downward component of the R-wave is sensitive to chloride movement through $GABA_A$ receptor channels, but not all the different types of Cl^- channels in *pars intermedia* cells modulate the R-wave in a similar fashion; and (c) the R-wave is biphasic and its full time-course is measured in minutes, implying not only the opening

of $GABA_A$ receptor channels, but also the participation of some other process(es) which may be involved in the restoration of the steady-state chloride concentration and, as such, might contribute to cell volume restoration (swelling) following GABA-induced shrinkage.[31,32]

Volume regulation and ionic homeostasis are among the functions of Cl^--conducting channels, including the $GABA_A$ receptors, and these functions are often carried out in concert with the expression and functional activity of ion transporters. We suspected[10] that GABA transporters were also involved in the *pars intermedia*, thereby influencing the R-wave, the long-term intrinsic optical signal recorded from *pars intermedia* cells. GABA transporters accomplish the reuptake of GABA *via* Na^+ and Cl^- concentration gradients. The Na^+ gradient is maintained primarily by the activity of a Na^+-K^+-ATPase and, therefore, is sensitive to oxygen levels. Of the four GABA transporters[33] the GAT1 exclusively transports GABA,[34] while the other transporters are also able to transport β-alanine and taurine.[35] In our experiments we saw no effect of taurine (data not shown), but observed changes caused by application of nipecotic acid,[26,36] and of the specific GAT1 inhibitor NO711.[24,25] The specificity of NO711 to GAT1 allowed us to speculate that, of the four GAT subtypes, GAT1 plays the primary role in the restoration of the volume equilibrium in the mouse *pars intermedia*. NO711 intensifies the GABA-induced intrinsic optical change, in contrast to $GABA_A$ receptor blockers such as picrotoxin and bicuculline (Figs. 3 and 4; see also Ref. 10).

The effect of nipecotic acid (see Fig. 5) provides additional evidence that the R-wave of the light-scattering signal is related to GAT1. At low concentration (100 μM), the drug induces a change in the R-wave similar to that induced by NO711.[26] At a much higher concentration (4 mM), however, the effect on the downward slope of the R-wave is amplified beyond what would have been expected solely from the GABA which, released by electrical stimulation, would have remained in the cleft due to block of GAT1. Instead, this result confirms that, at high concentration, nipecotic acid acts as a GABA-agonist for the $GABA_A$ receptors (e.g., see Fig. 2).[26]

If the physiological experiments (see Figs. 2–5) demonstrate GAT1's capacity to affect the R-wave of the light-scattering change by altering the concentration of GABA in the cleft, Fig. 6 provides evidence that GAT1 is expressed in the place that is most appropriate to this function. With these data, we have provided support for our initial hypothesis that the interplay between Cl^- movement through $GABA_A$ receptors on the one hand, and GABA uptake by GAT1 on the other, define the biphasic nature of the R-wave. Additionally, we have established light-scattering measurement as a powerful, noninvasive tool for monitoring, in real time, dynamic cellular processes.

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